

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

005430.00002

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR

10/070666

INTERNATIONAL APPLICATION NO.
PCT/EP00/08778INTERNATIONAL FILING DATE
September 8, 2000PRIORITY DATE CLAIMED
September 10, 1999

TITLE OF INVENTION

NUCLEIC ACID WHICH IS OBTAINED FROM TETRAHYMENA AND WHICH CODES FOR A
DELTA-6-DESATURASE, THE PRODUCTION THEREOF AND USE

APPLICANT(S) FOR DO/EO/US

Matthias RUSING, Thomas KIY and Annette DOMINITZKI

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☒ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

PCT/RO/101 (4 pp.); PCT/IB/306 (1 p.); Letter dated Nov. 6, 2001 to the EPO Munich (4 pp.); Copy of WO 01/20000 published March 22, 2001 w/PCT/ISA/210; English Translation: Specification (38 pp.), Claims 1-16 (2 pp.), 14 sheets of Drawings; Sequence Listing 8 pp.; Submission of Sequence Listing w/10 p. Sequence Listing; PCT/IPEA/409 (6 pp.) w/Amended Sheets (Claims 1-14) (2 pp.); PCT/IPEA/408(6 pp.); PCT/IB/304 (1 p.)

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1.101) 10/070666		INTERNATIONAL APPLICATION NO. PCT/EP00/08778		ATTORNEY'S DOCKET NUMBER 005430.00002	
24. The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) : <input type="checkbox"/> Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00 <input checked="" type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00 <input type="checkbox"/> International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00 ENTER APPROPRIATE BASIC FEE AMOUNT =				CALCULATIONS PTO USE ONLY	
Surcharge of \$130.00 for furnishing the oath or declaration later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				\$890.00	
				\$0.00	
CLAIMS		NUMBER FILED		NUMBER EXTRA	RATE
Total claims		32 - 20 =		12	x \$18.00
Independent claims		13 - 3 =		10	x \$84.00
Multiple Dependent Claims (check if applicable).				<input type="checkbox"/>	
TOTAL OF ABOVE CALCULATIONS =				\$216.00	
<input type="checkbox"/> Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				\$840.00	
				\$0.00	
SUBTOTAL =				\$0.00	
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).				\$1,946.00	
TOTAL NATIONAL FEE =				\$0.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).				\$1,946.00	
TOTAL FEES ENCLOSED =				\$0.00	
				Amount to be refunded	\$
				charged	\$
a. <input type="checkbox"/> A check in the amount of _____ to cover the above fees is enclosed.					
b. <input checked="" type="checkbox"/> Please charge my Deposit Account No. 19-0733 in the amount of \$1,946.00 to cover the above fees. A duplicate copy of this sheet is enclosed.					
c. <input checked="" type="checkbox"/> The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 19-0733 A duplicate copy of this sheet is enclosed.					
d. <input type="checkbox"/> Fees are to be charged to a credit card. WARNING: Information on this form may become public. Credit card information should not be included on this form. Provide credit card information and authorization on PTO-2038.					
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.					
SEND ALL CORRESPONDENCE TO:					
BANNER & WITCOFF, LTD. 28 State Street, 28th Floor Boston, MA 02109					
<i>Signature</i> SIGNATURE Peter D. McDermott NAME 29,411 REGISTRATION NUMBER March 8, 2002 DATE					

Rec'd PCT/PTO 09 SEP 2002

PATENT #

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Attorney Docket No. 005430.00002)

5

Applicants: Rusing et al. Paper No:
U.S. Serial No.: 10/070,666 Group Art Unit: TBA
Filed: March 08, 2002 Examiner: TBA
Title: NUCLEIC ACID WHICH IS OBTAINED FROM TETRAHYMENA
AND WHICH CODES FOR A DELTA-6-DESATURASE, THE
PRODUCTION THEREOF AND USE

SECOND PRELIMINARY AMENDMENT

Dear Sir:

Prior to examining the above-referenced application, kindly enter the amendments and remarks shown below.

AMENDMENTS

IN THE SPECIFICATION

Kindly replace the sequence listing filed with the original application with the following amended sequence listing listed on the attached pages.

PATENT

SEQUENCE LISTING

<110> Aventis Research & Technologies GmbH & Co KG

<120> Nucleic Acid Which is Obtained from Tetrahymena and Which Codes for a delta 6-desaturase, the Production Thereof and Use

<130> Banner & Witcoff Attorney Docket Number 005430.00002; National Phase

<140> US/10/070,666

<141> 2002-03-08

<160> 19

<170> PatentIn version 3.1

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atttgtttat tgatgagaag taagatttga ctgaatattt cagaacactc cattctaagt
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Second Preliminary Amendment

U.S. 10/070,666

Page 2 of 14

PATENT

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PATENT

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Thr Leu His Ser Lys Gln Ala Leu Lys Ile Leu Lys Ser Phe Pro Lys
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Asn Ile Leu Lys Asp Glu Asp Ile Gln His Asp Tyr Lys Leu Trp Gln
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Second Preliminary Amendment

U.S. 10/070,666

Page 4 of 14

PATENT

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Second Preliminary Amendment

U.S. 10/070,666

Page 5 of 14

PATENT

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Second Preliminary Amendment

U.S. 10/070,666

Page 6 of 14

PATENT

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Second Preliminary Amendment

U.S. 10/070,666

Page 7 of 14

PATENT

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PATENT

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PATENT

REMARKS

I. Status of the Claims

The foregoing amendments are intended to replace the original sequence listing with amended sequence listing. No new matter has been added by these amendments. An electronic version of the amended sequence listing is submitted with this paper.

Applicants respectfully request entry of the foregoing amendments and examination of the application.

Respectfully submitted,
Rusing et al.

Christopher R. Rusing (Reg. No. 47022)

for Peter D. McDermott

Peter D. McDermott
Attorney for Applicants
Reg. No. 29,411

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Telephone: (617) 227-7111
Facsimile: (617) 227-4399

CERTIFICATE OF EXPRESS MAILING

I hereby certify that this correspondence is being deposited with the United States Postal Service as Post Office To Addressee Express Mail No. EV066773938US in an envelope addressed to: Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231 on: September 9, 2002

Rachelle Chery
Rachelle Chery

9/9/02
Date

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
(Attorney Docket No. 005430.00002)

BOX PCT

Applicants: Rusing et al.

U.S. National Phase Application of
PCT/EP00/08778

Filed September 8, 2000

Paper No:

U.S. Serial No.: TBA

Group Art Unit: TBA

Filed: Herewith

Examiner: TBA

Title: NUCLEIC ACID WHICH IS OBTAINED FROM TETRAHYMENA
AND WHICH CODES FOR A DELTA-6-DESATURASE, THE
PRODUCTION THEREOF AND USE

PRELIMINARY AMENDMENT

Dear Sir:

Prior to examining the above-referenced application, kindly enter the amendments and remarks shown below. An attachment entitled "VERSION SHOWING CHANGES MADE" is included with this paper showing the amendments made to the claims.

AMENDMENTS

IN THE SPECIFICATION

Kindly add the following paragraph on page 1 of the specification after the word "Description."

--This is a U.S. National Phase Application Under 35 U.S.C. § 371 and Applicants herewith claim the benefit of priority of PCT/EP00/08778 filed September 8, 2000, which

was published Under PCT Article 21(2) in German and Application No. 199 43 270.8 filed in Germany on September 10, 1999.--

IN THE CLAIMS

Kindly cancel claim 16 without prejudice to Applicants' right to pursue the claim in one or more other patent applications.

Kindly amend claims 1 and 3-15 as follows:

Claim 1. (Amended) A nucleic acid encoding *Tetrahymena* delta-6-desaturase and comprising at least 8 nucleotides from SEQ ID NO.: 1.

Claim 3. (Amended) A nucleic acid as claimed in claim 1, which is obtained from *Tetrahymena thermophila*.

Claim 4. (Amended) A nucleic acid as claimed in claim 1 which is DNA.

Claim 5. (Amended) A nucleic acid as claimed in claim 1, which is a DNA having a nucleic acid sequence as depicted in SEQ ID. NO.: 1 from position 33 to position 1091.

Claim 6. (Amended) A nucleic acid as claimed in claim 1 comprising one or more noncoding sequences.

Claim 7. (Amended) An isolated nucleic acid comprising at least 8 nucleotides from SEQ ID NO.: 3.

Claim 8. (Amended) A vector comprising a nucleic acid comprising SEQ ID NO.: 1 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 1.

Claim 9. (Amended) The vector of claim 8, wherein the nucleic acid is functionally combined with a constitutive promoter.

PATENT

Claim 10. (Amended) A process for preparing a nucleic acid comprising SEQ ID NO.: 1 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 1, the process comprising chemically synthesizing the nucleic acid comprising SEQ ID NO.: 1 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 1.

Claim 11. (Amended) A polypeptide comprising an amino acid sequence comprising at least 6 amino acids from SEQ ID NO.: 2.

Claim 12. (Amended) A process for preparing a polypeptide comprising SEQ ID NO.: 2 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 2, the process comprising expressing a nucleic acid comprising SEQ ID NO.: 1 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 1 in an expression system.

Claim 13. (Amended) A specific antibody directed against a polypeptide comprising SEQ ID NO.: 2 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 2.

Claim 14. (Amended) A transgenic, nonhuman organism comprising a nucleic acid comprising SEQ ID NO.: 1 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 1.

Claim 15. (Amended) The transgenic organism of claim 14 in which the transgenic organism is a plant or a ciliate.

Kindly add the following new claims 17-31:

PATENT

Claim 17. (NEW) The nucleic acid of claim 1 wherein the *Tetrahymena* delta-6-desaturase comprises a polypeptide comprising SEQ ID NO.: 2 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 2.

Claim 18. (NEW) The nucleic acid of claim 1 comprising functional variants having at least 70% sequence identity with SEQ ID NO.: 1.

Claim 19. (NEW) The nucleic acid of claim 4 in which the DNA is double-stranded DNA.

Claim 20. (NEW) The vector of claim 8 in which the vector is an expression vector.

Claim 21. (NEW) The vector of claim 8 wherein the nucleic acid is functionally combined with an inducible promoter.

Claim 22. (NEW) The vector of claim 9 wherein the nucleic acid is functionally combined with an inducible promoter.

Claim 23. (NEW) The vector of claim 9 wherein the nucleic acid further comprises a termination signal.

Claim 24. (NEW) The vector of claim 21 wherein the nucleic acid further comprises a termination signal.

Claim 25. (NEW) The vector of claim 22 wherein the nucleic acid further comprises a termination signal.

Claim 26. (NEW) A process for preparing a nucleic acid comprising SEQ ID NO.: 1 or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 1, the process comprising isolating the nucleic acid from a gene library using a probe which hybridizes to the nucleic acid.

Claim 27. (NEW) The process of claim 20 in which the expression vector is in a host organism.

Claim 28. (NEW) A method of enriching delta-6-desaturase dependent fatty acids in ciliates, the method comprising:

inserting a vector comprising a nucleic acid comprising SEQ ID NO.: 1, or functional variants thereof having at least 70% sequence identity with SEQ ID NO.: 1, into a ciliate; and

expressing the nucleic acid to enrich delta-6-desaturase dependent fatty acids in the ciliate.

Claim 29. (NEW) The method of claim 28 in which the vector comprises at least one inducible promoter.

Claim 30. (NEW) An isolated and purified nucleic acid consisting essentially of SEQ ID NO.: 1 or functional variants thereof.

Claim 31. (NEW) An isolated and purified nucleic acid consisting essentially of SEQ ID NO.: 3 or functional variants thereof.

Claim 32. (NEW) An isolated and purified polypeptide consisting essentially of SEQ ID NO. 2 or functional variants thereof.

Claim 33. (NEW) A nucleic acid as claimed in claim 1 which is RNA.

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PATENT

REMARKS

I. Status of the Claims

The subject application is the U.S. national stage of PCT Application No. PCT/EP00/08778. The claims of the application are an English translation of the claims (as amended during PCT processing) of PCT/EP00/08778.

The foregoing amendments put the claims in proper and more traditional format for prosecution in the U.S. Patent Office. Claims that depended from multiple claims have been amended to depend from only a single claim. New claims 17-31 have been added to claim the subject matter originally claimed in claims which depended from multiple claims. No new matter has been incorporated by these amendments.

Applicants respectfully request entry of the foregoing amendments and examination of the application.

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Examiner: TBA

Title: **Structure of the Delta-6-Desaturase Gene**

VERSION SHOWING CHANGES MADE

Claim 1. (Amended) A nucleic acid encoding *Tetrahymena* delta-6-desaturase ~~having an amino acid sequence as depicted in SEQ ID NO.: 2, or a functional variant thereof having at least 70% sequence identity with SEQ ID NO.:2, and parts thereof containing comprising at least 8 nucleotides ,with from~~ SEQ ID NO.: 1 ~~being part of the claim.~~

Claim 3. (Amended) A nucleic acid as claimed in claim 1 or 2, which is obtained from *Tetrahymena thermophila*.

Claim 4. (Amended) A nucleic acid as claimed in claims 1-3 which is a DNA or RNA, preferably a double-stranded DNA.

Claim 5. (Amended) A nucleic acid as claimed in ~~one of~~ claims 1 to 4, which is a DNA having a nucleic acid sequence as depicted in SEQ ID. NO.: 1 from position 33 to position 1091.

Claim 6. (Amended) A nucleic acid as claimed in ~~one of claims 1 to 5, which contains~~
comprising one or more noncoding sequences.

Claim 7. (Amended) An isolated nucleic acid ~~as claimed in one of claim 1 to 6 as~~
~~depicted in SEQ ID NO.: 3 or a functional variant thereof, and parts thereof containing~~
comprising at least 8 nucleotides with from SEQ ID NO.: 3 being part of the claim.

Claim 8. (Amended) A nucleic acid ~~as claimed in one of claims 1-7, which is contained~~
~~in a vector, preferably an expression vector~~ comprising a nucleic acid comprising SEQ
ID NO.: 1 or functional variants thereof having at least 70% sequence identity with SEQ
ID NO.: 1.

Claim 9. (Amended) ~~An~~The ~~expression vector as claimed in of~~ claim 8, wherein the
nucleic acid is functionally combined with a constitutive ~~and/or inducible~~ promoter and
~~optionally with a termination signal.~~

Claim 10. (Amended) A process for preparing a nucleic acid ~~as claimed in one of claim~~
~~1-7, which comprises~~ comprising SEQ ID NO.: 1 or functional variants thereof having at
least 70% sequence identity with SEQ ID NO.: 1, the process comprising chemically
synthesizing the nucleic acid comprising SEQ ID NO.: 1 or functional variants thereof
having at least 70% sequence identity with SEQ ID NO.: 1 ~~or isolating it from a gene~~
~~library using a probe.~~

Claim 11. (Amended) A polypeptide ~~having~~comprising an amino acid sequence as
~~depicted in~~ comprising at least 6 amino acids from SEQ ID NO.: 2 or a functional variant
~~thereof, and parts thereof containing at least 6 amino acids.~~

Claim 12. (Amended) A process for preparing a polypeptide ~~as claimed in claim 11~~
comprising SEQ ID NO.: 2 or functional variants thereof having at least 70% sequence
identity with SEQ ID NO.: 2, which comprises the process comprising expressing a
nucleic acid in one of claims 1-7 comprising SEQ ID NO.: 1 or functional variants

thereof having at least 70% sequence identity with SEQ ID NO.: 1 in an suitable
expression system ~~or host organism~~.

Claim 13. (Amended) A specific antibody ~~which is~~ directed against a polypeptide as
~~elaimed in claim 11~~ comprising SEQ ID NO.: 2 or functional variants thereof having at
least 70% sequence identity with SEQ ID NO.: 2.

Claim 14. (Amended) A transgenic, nonhuman organism ~~which harbors~~ comprising a
nucleic acid as elaimed in one of claims 1 to 10 comprising SEQ ID NO.: 1 or functional
variants thereof having at least 70% sequence identity with SEQ ID NO.: 1.

Claim 15. (Amended) A ~~The~~ transgenic organism as ~~elaimed in~~ of claim ~~11~~ 14 in which is
~~selected from the transgenic organism is a~~ plants or a ciliates.

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PCT/EP00/08778

TETRAHYMENA NUCLEIC ACID ENCODING A
DELTA-6-DESATURASE, ITS PREPARATION AND USE

Description

5

The present invention relates to a Tetrahymena delta-6-desaturase, to its encoding nucleic acid and to its preparation and use.

10 The invention relates to a Tetrahymena nucleic acid which encodes a ciliate-specific delta-6-desaturase which is involved in the biosynthesis of commercially valuable polyunsaturated fatty acids (what are termed PUFAs, i.e. polyunsaturated fatty acids) in eukaryotes.

15 In this connection, the nucleic acids according to the invention, and the polypeptides which can be obtained from them, exhibit surprisingly little sequence identity with other known natural desaturases. The invention furthermore relates to the use of the nucleic

20 acids for overexpression in eukaryotes, in particular ciliates, preferably Tetrahymena, particularly preferably *Tetrahymena thermophila*, with the aim of specifically modifying the fatty acid spectrum, in particular with the aim of increasing PUFA formation.

25

The nucleic acids according to the invention can be obtained from ciliates, preferably from Tetrahymena, particularly preferably from *Tetrahymena thermophila*, a GLA-producing organism having a very high content of

30

GLA.

Figure 1 shows a general scheme of the biosynthesis of PUFAs, and the enzymes involved, in eukaryotes (modified after Gill & Valivety, Trends Biotechnol.

35 1997, 15:401-409). The conversion of stearic acid (18:0) to oleic acid (18:1 Δ^9) is catalyzed by a delta-9-desaturase. Oleic acid is converted by a delta-12-desaturase into linoleic acid (18:2 $\Delta^9,12$; abbreviated

to LA), which is in turn converted by a delta-6-desaturase into γ -linolenic acid (18:3, Δ 6,9,12; abbreviated to GLA) or by a delta-15-desaturase into α -linolenic acid (18:3 Δ 9,12,15; abbreviated to ALA). The fatty acids are extended by elongases, as a result of which dihomogamma-linolenic acid (20:3 Δ 8,11,15; abbreviated to DGLA) is, for example, formed from γ -linolenic acid, with the dihomogamma-linolenic acid in turn being converted by a delta-5-desaturase into arachidonic acid (20:4 Δ 5,8,11,15; abbreviated to ARA), which is a direct precursor of physiologically active eicosanoids, such as prostaglandins, prostacyclins, thromboxanes and leukotrienes. The conversion of LA to GLA by delta-6-desaturase has been found to be a limiting step in the formation of the PUFAs (termed delta-6-unsaturated fatty acids in that which follows) which are derived from GLA (Huang YS & Mills DE (1996) γ -Linolenic acid. Metabolism and its role in nutrition and medicine. AOCS Press, Champaign, Illinois, 1996).

While an enzyme having delta-6-desaturase activity is known to be present in *Tetrahymena setosa* and *T.pyriformis* (Peng, Y.M. and Elson, C.E. (1971) J. Nutr. 101, 1177-1184), a homogeneous protein which is derived from a ciliate and which possesses such an activity has not previously been made available (e.g. Koll, M. and Erwin, J.A. (1990) J. Protozool. 37(3), 229-237).

Since vertebrates are unable to insert any double bonds behind position 9 in fatty acids, unsaturated fatty acids such as LA and ALA are essential nutrients which vertebrates cannot synthesize (see Figure 1) and, in the diet, are principally derived from plant sources. In mammals, a delta-6-desaturase can convert LA into GLA, which is a precursor of ARA, which is in turn an essential precursor of most prostaglandins. The formation of stearidonic acid (18:4 Δ 6,9,12,15), which

WO 01/20000

PCT/EP00/08778

- 3 -

is a precursor of EPA, from ALA is likewise catalyzed by means of delta-6-desaturase. Delta-6-desaturase is consequently the first essential step in the biosynthesis of the eicosanoids (see Figure 1).

5

It has been found that, in mammals, the activity of delta-6-desaturase can be impaired by factors such as alcohol consumption, stress, deficient nutrition and aging processes (Huang & Mills, 1996; Horrobin (1990) Rev. Contemp. Pharmacother. 1:1-45; Bolton-Smith C et al. (1997) Eur. J. Clin. Nutr. 51:619-624; Leventhal LT et al. (1993) Ann. Intern. Med. 119:867-873). This leads to an insufficient supply of GLA and thus ultimately a deficiency in the molecules derived from GLA, such as ARA and the physiologically important eicosanoids which are formed therefrom, since, as has already been mentioned, the formation of GLA from LA by delta-6-desaturase is a limiting step in PUFA synthesis (Brenner RR (1976) Adv. Exp. Med. Biol. 83:85-101; Nakahara T et al. (1993) J. Jpn. Oil Chem. Soc. 42:242-253; Chapkin, RS (1998) Reappraisal of the essential fatty acids. In: Fatty acids in food and their health implications, 2nd ed. (Chow CK, ed) Marcel Dekker, New York, NY). Supplying GLA can both compensate for a reduced endogenous level of delta-6-unsaturated fatty acids and cover an increased requirement for these fatty acids (Horrobin (1990)). The uptake of GLA via the diet is therefore advantageous for the biosynthesis of GLA-derived molecules (Fan, YY & Chapkin, RS (1998) J. Nutr. 128:1411-1414).

The finding that GLA exerts many different positive influences on the human body has by now been underpinned by a large number of scientific studies. Thus, clinical studies have demonstrated the positive effect of GLA on, for example, atopic eczema (Shimaski, H: PUFA content and effect of dietary intake of γ -linolenic acid-rich oil on profiles of n-6, n-3

metabolites in plasma of children with atopic eczema. J. Clin. Biochem. Nutr. (1995), 19(3), 183-192), rheumatoid arthritis (Zurier TB, Rossetti RG, Jacobson EW, DeMarco DM, Liu NY, Temming JE, White BM, Laposata M (1996) γ -Linolenic acid treatment of rheumatoid arthritis: A randomized, placebo-controlled trial. Arthritis Rheum. 39(11) 1808-1817), atherosclerosis (Leng GC, Lee AJ, Fowkes FGR, Jepson RG, Lowe GDO, Skinner ER, Mowat BF, Randomized controlled trial of γ -linolenic acid and eicosapentaenoic acid in peripheral arterial disease. Clinical Nutrition, (1998) 17/6 265-271), diabetic neuropathy (Pfeifer MA, Schumer MP (1995) Clinical trials of diabetic neuropathy: past, present, and future. Diabetis 44(12) 1355-61), migraine (Wagner W, Nootbaar-Wagner U (1997) Prophylactic treatment of migraine with γ -linolenic and alpha-linolenic acids, Cephalalgia 17/2 127-130), schizophrenia (Vaddadi, KS (1982) Some observations on the use of prostaglandin E1 precursor in the treatment of schizophrenia. Biol. Aspects Schizophr. Addict. 183-91. Publisher: Wiley, Chichester, UK) and cancer (Kairemo KJA, Jekunen AP, Korppi-Tommola ET, Pyrhonen SO (1997) Effects of lithium γ -linolenate on the perfusion of liver and pancreatic tissues in pancreatic cancer. Anticancer Research 17/5 B 3729-3736). These studies achieved both a statistical improvement and a clinically significant improvement in the clinical picture. The effect of GLA in this connection is due, in particular, to the formation of eicosanoids (prostaglandins, prostacyclins, thromboxanes and leukotrienes), for whose biosynthesis GLA is a precursor molecule (Figure 1).

Because of these positive properties, a broad spectrum of application exists for GLA in the pharmaceutical industry, the cosmetics industry, the animal feed industry and the foodstuffs industry (Horrobin (1990),

Horrobin (1992) Prog. Lipid Res. 31:163-194; Chapkin (1998), Fan & Chapkin (1998)).

Most PUFAs found in humans and animals are either
5 derived directly from the diet or formed by the
conversion, by desaturases and elongases, of the
essential fatty acids which are supplied via the diet.
For this reason, the PUFA biosynthesis genes which are
10 derived from organisms in which these PUFAs occur
naturally are of great commercial interest. The
commercial production of PUFAs can be achieved in
organisms or cells by expressing these genes in the
systems in a specific and functional manner. For this
reason, a requirement exists for genes encoding
15 desaturases and elongases involved in PUFA biosynthesis
and for using these genes for obtaining PUFAs and PUFA
oils commercially by means of reliable and economic
methods.

20 None of the commercially employed oilseeds produces
GLA. On the contrary, GLA only occurs in the oil
derived from seeds of various plants such as common
evening primrose (*Oenothera biennis*, approx. 10% GLA),
borage (*Borago officinalis*, approx. 23%) and
25 blackcurrant (*Ribes nigrum*, approx. 18%). In addition
to this, a variety of microorganisms, such as the fungi
Mucor and *Mortierella* (up to approx. 25%), the blue-
green alga *Spirulina* (approx. 12-18%), and others, are
known to be sources of GLA. Ciliates such as
30 *Tetrahymena* (up to 47%; Hill, DL (1972) The
biochemistry and physiology of *Tetrahymena*. Chapter 3,
46-73. Academic press, New York, London; Erwin, J &
Bloch, K (1963) J. Biol. Chem. 238:1618-1624) have been
reported to be a particularly rich source of GLA.
35 Phillips & Huang provide a good review of the natural
sources of GLA (Phillips JC, Huang YS (1996) Natural
sources and biosynthesis of γ -linolenic acid: an
overview. 1-13 in: γ -linolenic acid. Metabolism and its

role in nutrition and medicine, Huang YS, Mills DE (eds.) AOCS Press, Champaign, Illinois, 1996).

However, the commercial isolation of GLA from these
5 natural sources is associated with a number of
disadvantages. Both the quality and the quantity of the
oils obtained from these organisms vary and, in some
cases, the composition of the oils is very
heterogeneous, making it necessary to use elaborate and
10 expensive purification steps for the purpose of
concentrating the GLA. In addition to this, cultivating
GLA-containing plants is not a very economical process
(Hansen CE et al. (1991) J. Sci. Food Agric. 54:309-
312). It has been found that the space/time yield for
15 obtaining GLA-containing oil is markedly better in some
GLA-producing microorganisms than in higher plants. For
this reason, the fermentative preparation of GLA using
microorganisms offers a promising alternative to other
sources of GLA. The fatty acid spectrum of many
20 microorganisms is frequently rather simple as compared
with that of higher organisms, a feature which offers
great advantages during purification. Furthermore,
fermentative preparation is not dependent on external
factors such as weather, supply of nutrients, etc. In
25 addition, PUFAs which are prepared in this way are to a
large extent free of contaminants which can be
attributed, for example, to environmental pollution. A
further advantage is that, in contrast to GLA derived
from natural sources, GLA which is isolated by
30 fermentative processes is not subject to any
fluctuations in availability.

There have already been attempts to develop systems for
isolating GLA by fermentation (Ratledge C (1993) Trends
35 Biochem. 11:278-284; Ratledge C (1989) Biochem. Soc.
Trans. 17:1139-1141; Gosselin Y et al. (1989)
Biotechnol. Lett. 11:423-426; WO86/03518). However,
when microorganisms are to be used for commercially

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A homology comparison was used to identify the protein sequence which is derived from the nucleic acid sequence (SEQ ID No.: 1), and which is depicted in SEQ ID No.: 2. as being a delta-6-desaturase. The BLASTP function (Altschul et al. (1997) *Nucleic Acids Res.* 25:3389-3402) was used for the homology comparison. Desaturases, in particular delta-6-desaturases (E.C. 1.14.99.25; linoleoyl-CoA desaturase) were identified from the databases as being homologous proteins (see Figure 2). In this connection, the known delta-6-desaturases exhibit a maximum identity of 25% with the polypeptide sequence according to the invention (see Figures 3A-3E). A multiple alignment of various known delta-6-desaturases with the polypeptide sequence according to the invention is shown in Figure 4. The homologies are to be found, in particular, in conserved domains such as the histidine boxes (Los & Murata, 1998. *Biochem. Biophys. Acta* 1394: 3-15; Shanklin, J et al. 1997. *Proc. Natl. Acad. Sci. USA* 92, 6743-6747). In addition to this, it was possible to identify a cytochrome b5 domain as in other eukaryotic delta-6-desaturases (Lederer, F. (1994) *Biochimie* **76**, 674-692; Cho et al. *J. Biol. Chem.* 1999, 274(1): 471-477). Although the polypeptide sequence according to the invention can be identified as being a delta-6-desaturase, it differs substantially from other delta-6-desaturases. It is particularly striking that, with 352 amino acids, the sequence is around 20% shorter than other eukaryotic delta-6-desaturases. In addition to this, the sequence exhibits a large number of unique differences in strongly conserved regions. Thus, the

25 According to the present invention, the term
"functional variant" is understood as meaning a nucleic
acid which is related functionally to the Tetrahymena
delta-6-desaturase. Examples of related nucleic acids
30 are, for example, nucleic acids from other ciliate
cells, or allelic or degenerate variants. The invention
likewise encompasses functional variants of the nucleic
acids according to the invention which, because of the
unusual codon usage (see: Wuitschick JD, Karrer KM
35 (1999) Analysis of genomic G + C content, codon usage,
initiator codon context and translation termination
sites in *Tetrahymena thermophila*. J. Eukaryot. Micro-
biol. 1999 46(3):239-47), require an adaptation of the

nucleic acids according to the invention in selected expression systems. On the one hand, this relates to the replacement of the codons TAA and TAG, which encode glutamine in ciliates and which are stop codons in most other expression systems, with CAA and CAG. In addition to this, the skilled person is familiar with optimally adapting the nucleic acids according to the invention to the given particular codon preference (what is termed codon usage) of different expression systems.

5 The nucleic acids can be modified in a known manner by specifically substituting bases, or else the nucleic acid which is required is obtained from artificially prepared oligonucleotides. The adaptation of the sequence can be carried out, for example, on the basis of the known codon usage tables (e.g. on the Internet: Codon usage tabulated from Genbank: <http://www.dna.affrc.go.jp/~nakamura/CUTG.html>) for the preferred expression systems. The present invention also encompasses variants of nucleic acids which only contain parts of the nucleic acid.

According to the present invention, the term "variants" is understood, in a broader sense, as meaning nucleic acids which exhibit a homology, in particular a sequence identity, of approx. 60%, preferably of approx. 75%, in particular of approx. 90% and especially of approx. 95%.

The parts or fragments of the nucleic acids according to the invention can, for example, be used for preparing individual epitopes, as probes for identifying other functional variants, or as antisense nucleic acids. For example, a nucleic acid comprising at least approx. 8 nucleotides is suitable for use as an antisense nucleic acid, while a nucleic acid comprising at least approx. 15 nucleotides is suitable for use as a primer in the PCR method, a nucleic acid comprising at least approx. 20 nucleotides is suitable

for identifying other variants, and a nucleic acid comprising at least approx. 100 nucleotides is suitable for use as a probe.

5 In another preferred embodiment, the nucleic acid according to the invention contains one or more noncoding sequences (UTR inter alia). The noncoding sequences are, for example, intron sequences or regulatory sequences, such as promoter or enhancer
10 sequences, for the controlled expression of the delta-6-desaturase-encoding gene. The invention therefore relates to a nucleic acid according to the invention as depicted in SEQ ID No.: 3, which nucleic acid can be isolated from *Tetrahymena thermophila* and represents
15 the genomic sequence of the delta-6-desaturase together with introns, promoter and UTRs.

In a further embodiment, the nucleic acid according to the invention is therefore contained in a vector,
20 preferably in an expression vector.

The expression vectors can, for example, be prokaryotic or eukaryotic expression vectors. An example of a prokaryotic expression vector for expression in *E. coli*
25 is the T7 expression vector pGM10 (Martin, 1996), which encodes an N-terminal Met-Ala-His6 tag, which enables the expressed protein to be advantageously purified by way of an Ni²⁺-NTA column. Examples of eukaryotic expression vectors which are suitable for expression in
30 *Saccharomyces cerevisiae* are the vectors p426Met25 or p426GAL1 (Mumberg et al. (1994) Nucl. Acids Res., 22, 5767) while examples of such vectors which are suitable for expression in insect cells are baculovirus vectors, as disclosed in EP-B1-0127839 or EP-B1-0549721, and for
35 expression in mammalian cells SV40 vectors, which are available generally.

In general, the expression vectors also contain regulatory sequences which are suitable for the host cell, such as the trp promoter for expression in E. coli (see, for example, EP-B1-0154133), the ADH-2 promoter for expression in yeast (Russel et al. (1983), J. Biol. Chem. 258, 2674), the baculovirus polyhedrin promoter for expression in insect cells (see, for example, EP-B1-0127839) and the early SV40 promoter or LTR promoters derived, for example, from MMTV (mouse mammary tumor virus; Lee et al. (1981) Nature, 214,228).

The vectors described, for example, by Gaertig et al. ((1999) Nature Biotech. 17: 462-465) or Gaertig & Kapler ((1999) Methods in Cell Biol. 62:485-500) are suitable for the transformation of, and expression in, Tetrahymena.

The nucleic acids according to the invention can be synthesized, for example chemically, for example in accordance with the phosphotriester method, using the sequences disclosed in SEQ ID No.: 1 and 3 or using the peptide sequence disclosed in SEQ ID No.: 2 and enlisting the genetic code (see, for example, Uhlman, E. & Peyman, A. (1990) Chemical Reviews, 90, 543, No. 4). Another possibility of obtaining the nucleic acids according to the invention is to use a suitable probe to isolate them from a suitable gene library which has been prepared from an organism which possesses delta-6-desaturase activity (see, for example, Sambrook, J. et al. (1989) Molecular Cloning. A laboratory manual. 2nd edition, Cold Spring Harbor, New York). Examples of suitable probes are single-stranded DNA fragments having a length of from approx. 100 to 1000 nucleotides, preferably having a length of from approx. 200 to 500 nucleotides, in particular having a length of from approx. 300 to 400 nucleotides, whose sequence can be derived from the nucleic acid sequence depicted in SEQ ID No.: 1 or 3.

For this reason, the invention likewise relates to a process for preparing a nucleic acid according to the invention, with the nucleic acid being synthesized 5 chemically or being isolated from a gene library using a probe.

The present invention furthermore also relates to the polypeptide itself having an amino acid sequence as depicted in SEQ ID No.: 2, or a functional variant thereof, and parts thereof containing at least six amino acids, preferably containing at least 12 amino acids, in particular containing at least 65 amino acids and especially containing at least 150 amino acids (termed "polypeptide according to the invention" in that which follows). For example, a polypeptide of approx. 6-12 amino acids in length, preferably of approx. 8 amino acids in length, can contain an epitope which, after having been coupled to a carrier, can be used for preparing specific polyclonal or monoclonal antibodies (in this regard, see, for example, US 5,656,435). Polypeptides having a length of at least approx. 65 amino acids can also be used directly, without any carrier, for preparing polyclonal or monoclonal antibodies.

Within the meaning of the present invention, the term "functional variant" is understood as meaning polypeptides which are related functionally to the peptide according to the invention, i.e. exhibit a delta-6-desaturase activity.

In a broader sense, it is also understood as meaning polypeptides which possess a sequence homology, in particular a sequence identity, of approx. 70%, preferably of approx. 80%, in particular of approx. 90%, especially of approx. 95%, with the polypeptide

having the amino acid sequence as depicted in SEQ ID No.: 2.

Preference is furthermore given to polypeptides which possess conserved regions of histidine boxes and a cytochrome b5 domain. Particular preference is given to polypeptides according to the invention which contain an HHFEP motif.

10 The term "functional variant" furthermore also includes deletions of the polypeptide in the range of approx. 1-60, preferably of approx. 1-30, in particular of approx. 1-15, especially of approx. 1-5, amino acids. For example, the first amino acid methionine can be
15 missing without the function of the polypeptide being significantly altered. The term "functional variant" also includes fusion proteins which contain the above-described polypeptides according to the invention, with the fusion proteins themselves already possessing the
20 function of a delta-6-desaturase or only being able to gain the specific function after the fusion moiety has been eliminated. These fusion proteins especially include fusion proteins containing a constituent moiety of, in particular, non-ciliate sequences of approx. 1-200, preferably approx. 1-150, in particular approx. 1-100, especially approx. 1-50, amino acids. Examples of
25 non-ciliate peptide sequences are prokaryotic peptide sequences, derived, for example, from E. coli galactosidase, or what is termed a histidine tag, for example a Met-Ala-His6 tag. A fusion protein containing what is termed a histidine tag is particularly
30 advantageously suitable for purifying the expressed protein by way of metal ion-containing columns, for example by way of an Ni²⁺-NTA column. "NTA" stands for the chelator nitrilotriacetic acid (Qiagen GmbH, Hilden).

- 15 -

The parts of the polypeptide according to the invention represent epitopes, for example, which epitopes can be specifically recognized by antibodies.

5 The polypeptide according to the invention can, for example, be prepared, in accordance with methods which are well known to the skilled person, by expressing the nucleic acid according to the invention in a suitable expression system as already described above. Examples
10 of suitable host cells are the E. coli strains DH5, HB101 or BL21, the yeast strain *Saccharomyces cerevisiae*, the insect cell line *Lepidopteran*, for example obtained from *Spodoptera frugiperda*, or animal cells such as COS, Vero, 293 and HeLa, all of which are
15 available generally.

In particular, said parts of the polypeptide can also be synthesized with the aid of classical peptide synthesis (Merrifield technique). They are suitable, in particular, for obtaining antisera which can be used for screening suitable gene expression libraries in order, in this way, to obtain further functional variants of the polypeptide according to the invention.

25 The present invention therefore also relates to a process for preparing a polypeptide according to the invention, with a nucleic acid according to the invention being expressed in a suitable host cell and, where appropriate, isolated.

30 The present invention also relates to antibodies which react specifically with the polypeptide according to the invention, with either the abovementioned parts of the polypeptide themselves being immunogenic, or it
35 being possible for these parts to be made immunogenic, or for their immunogenicity to be increased, by coupling them to suitable carriers, such as bovine serum albumin.

Murphy (Current Opinion in Biotechnology (1999) 10:175-180) and Knutzon & Knauf (1998), for example, describe the problems and difficulties involved in specifically modifying the fatty acid spectrum in transgenic plants.

5 Because of the described high content of GLA in Tetrahymena, it is advantageous to use the PUFA biosynthesis genes from this organism for developing highly productive, commercially important strains by
10 means of recombinant methods. As a result of the possibility of being able to cultivate Tetrahymena well in mass culture and with a high cell density, it is additionally advantageous to use Tetrahymena itself for generating such highly productive, commercially
15 interesting strains by means of recombinant methods. Furthermore, with the aid of the nucleic acid according to the invention, it is also possible to use other organisms, besides Tetrahymena, for producing GLA and other delta-6-unsaturated fatty acids.

20 The preparation of GLA by fermenting Tetrahymena is particularly advantageous on account of the rather simple fatty acid spectrum as compared with higher organisms. In addition to this, a fermentative
25 production is not affected by external factors such as weather, supply of nutrients, etc. In addition, a product which is obtained in this way is to a large extent free of impurities which can be generated, for example in the case of products obtained from nature,
30 by environmental pollution.

It is possible to use the nucleic acids according to the invention, encoding a ciliate-specific Tetrahymena delta-6-desaturase, to generate transgenic organisms
35 which produce GLA and delta-6-unsaturated fatty acids or whose content of such fatty acids is substantially increased as compared with wild-type cells (in this case: *Tetrahymena thermophila*) (see Tables 1 and 2).

These transgenic organisms are preferably ciliates, particularly preferably Tetrahymena, which harbor the nucleic acid sequence according to the invention or express it in a functional manner. The expression of the desaturase in this way leads to a relative increase in delta-6-unsaturated fatty acids, or of secondary products derived therefrom, with this increase being based on a change in the concentration of enzymes and substrates involved in PUFA synthesis. The invention can be used in the commercial production of PUFAs, in particular of GLA and PUFAs which are derived therefrom, or other secondary products which can be derived from GLA, or of $\Delta 6$ -unsaturated fatty acids (see Figure 1: PUFA biosynthesis). In addition to GLA, it is also possible, in this way, by desaturating ALA, to prepare stearidonic acid (18:4 $\Delta 6,9,12,15$), for example, which is a raw material which is frequently employed industrially.

In a special embodiment, it is possible, by using the delta-6-desaturase-encoding nucleic acids according to the invention in functional combination with suitable regulatory sequences, to effect an increased expression of the enzyme and thereby to increase the GLA content in GLA-producing organisms or to produce GLA in LA-producing organisms. Oil-producing organisms, such as sunflower, colza and soybean, and also other organisms as well, are of particular interest in this connection. In addition to this, by simultaneously using, for example, a delta-12-desaturase (e.g. Sakurada et al. (1999) Eur. J. Biochem. 261:812-820, Okuley et al. Plant Cell (1994) 6(1) 147-58), it is possible to produce said PUFAs in organisms or cells which contain no LA or only a little LA. It is likewise possible to use a combination of the three desaturases which are involved in the formation of GLA, i.e. $\Delta 6$, $\Delta 9$ and $\Delta 12$, for producing GLA and delta-6-unsaturated fatty acids. Furthermore, combining with other genes involved in

PUFA biosynthesis (cf. Figure 1) constitutes another preferred embodiment of the invention, with the GLA and delta-6-unsaturated fatty acids being transformed by means of other enzymes for which GLA serves as a substrate, as a result of which it is possible to prepare, for example, ARA (20:4) and other molecules which are derived from GLA.

In addition, the invention can be used for preparing novel GLA-containing nutrient sources or nutrient sources which are rich in molecules (in particular PUFAs, e.g. ARA) which can be derived from GLA or other $\Delta 6$ -unsaturated fatty acids.

In addition, the present invention describes expression constructs which contain the delta-6-desaturase gene, or parts thereof, and also the functional combination of the delta-6-desaturase-encoding sequence with heterologous regulatory sequences.

The invention furthermore relates to the preparation of transgenic organisms having an elevated content of GLA (see Tables 1 and 2) by means of using the described delta-6-desaturase-encoding DNA sequence and the described functional constructs of the delta-6-desaturase gene.

A large number of well-established methods are known for isolating genomic DNA and mRNA and for preparing genomic libraries and cDNA libraries (e.g. in Sambrook et al. (1989) in Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, NY). Suitable vectors which contain the desaturase described in the invention, or parts thereof, can be prepared using methods which are known to the skilled person, as are described, for example, in Ausubel et al. (Ausubel et al. (1995), Current Protocols in Molecular Biology, Green

Publishing Associates, New York) and Sambrook et al. (1989).

Vectors which contain the delta-6-desaturase-encoding sequence can be introduced into cells by infection, transfection, electroporation, bombardment with particles and other methods. In the present instance, transformation is understood generally as meaning the introduction of foreign DNA into a cell. The methods for doing this are well established and can be carried out in a manner known to the skilled person (e.g. Sambrook et al. (1989), Potrykus I (1991) Annu. Rev. Plant Biol. Plant Mol. Biol. 42:205-225, Christou P (1993) Curr. Opp. Biotech. 4:135-141).

15 Vectors are also described, with these vectors containing the DNA sequence of the present invention, or parts of the sequence, in functional combination with promoters, or other regulatory elements, which are
20 active in a host cell. In a preferred embodiment, these regulatory elements are nucleic acid sequences which are functionally active in ciliates, in particular Tetrahymena, such as the promoters for histone H4, and α - and β -tubulin and others.

Organisms which express the described delta-6-desaturase-encoding sequence recombinantly are also described in the present invention. As a result, in addition to the possibility of producing Δ6-PUFAs in these organisms, there is also the possibility, for example, of isolating the recombinant delta-6-desaturase, or parts thereof, using standard methods of protein purification (e.g. Ausubel et al. (1995)). Vectors which can be used for expressing the delta-6-desaturase-encoding sequence in various organisms can be prepared in a manner known to the skilled person. Detailed information concerning suitable vectors can be found, for example, in Sambrook et al. (1989), Goeddel,

ed. (1990) Methods in Enzymology 185 Academic Press,
and Perbal (1988) A Practical Guide to Molecular
Cloning, John Wiley and Sons, Inc. These vectors
preferably contain sequence elements which influence
5 expression, such as promoters, enhancer elements,
upstream activating sequences, etc. Inducible and
constitutive promoters, or, for example, tissue-
specific promoters, are suitable for effecting
expression. The cauliflower mosaic virus (CaMV) 35S
10 promoter (Restrepo et al. (1990) Plant Cell 2 987), for
example, or, for example, promoters which are activated
in association with seed development, are suitable for
expression in plant cells.

15 The vectors which are employed are preferably shuttle
vectors (Wolk et al. (1984) Proc. Natl. Acad. Sci. USA,
1561-1565, Bustos et al. (1991) J. Bacteriol. 174:
7525-7533).

20 In a preferred embodiment, the nucleic acid according
to the invention is expressed in Tetrahymena under the
control of a strong promoter (such as the Tetrahymena
 β -tubulin promoter, Gaertig et al. (1999) Nature
Biotech.). The transformation can preferably be
25 effected in accordance with methods described by
Gaertig et al. (1999) Nature Biotech. 17:462-465 (or,
for example, by Gaertig & Gorovsky (1992) Proc. Natl.
Acad. Sci. USA 89:9196-9200). It is possible, for
example, to use the *Tetrahymena thermophila* α - or β -
30 tubulin promoters as regulatory elements for the
expression. The transformed Tetrahymena cells are
identified in selective media and then enriched and
cultured. It is possible to use standard methods to
isolate the lip(o)ids from the cells (e.g. Dahmer et
35 al., (1989) Journal of American Oil Chemical Society
66, 543). The methyl esters of the fatty acids can be
analyzed by gas chromatography.

Figure 4: Multiple alignment of the Tetrahymena polypeptide sequence depicted in SEQ ID No.: 2 with known desaturases. The histidine

Tetrahymena thermophila (strains B1868 VII, B2086 II, B*VI, CU427, CU428 and CU522, kindly provided by Dr. J. Gaertig, University of Georgia, Athens, GA, USA) were cultured in modified SPP medium (2% proteose peptone, 0.1% yeast extract, 0.2% glucose, 0.003% Fe-EDTA (Gaertig et al. (1994) PNAS 91:4549-4553)) or skimmed milk medium (2% skimmed milk powder, 0.5% yeast extract, 1% glucose, 0.003% Fe-EDTA) or MYG medium (2% skimmed milk powder, 0.1% yeast extract, 0.2% glucose, 0.003% Fe-EDTA) in the added presence of antibiotic solution (100 U of penicillin/ml, 100 µg of streptomycin/ml and 0.25 µg of amphotericin B/ml (SPPA medium)) at 30°C in a volume of 50 ml in 250 ml Erlenmeyer flasks while shaking (150 rpm).

WO 01/20000

PCT/EP00/08778

- 26 -

Plasmids and phages were replicated in *E. coli* XL1-Blue MRF', TOP10F' or JM109 and selected. The bacteria were cultured under standard conditions in LB or NZY medium containing antibiotics in standard concentrations
5 (Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, New York).

EXAMPLE 2:

10 Preparing a *Tetrahymena thermophila* cDNA library

Tetrahymena thermophila total RNA was isolated using the guanidine thiocyanate/phenol/chloroform method (Chomzynski & Sacchi (1987) Anal. Biochem. 161:156-
15 159). The mRNA was isolated from the total RNA using Oligotex-dT beads (Qiagen). The cDNA was synthesized using the Stratagene ZAP Express cDNA Synthesis and Cloning Kit. After ligating on the EcoR I adapter and digesting with Xho I, the DNA was separated on an
20 agarose gel and size-fractionated (S: 500 - 1500 bp, B: greater than 1500 bp). The DNA was isolated from the gel (Qiaquick gel extraction kit, QIAGEN) and ligated into ZAP Express vector, which had been cut with Eco RI and Xho I. The ligated DNA was packaged in vitro into
25 phages (Stratagene Gigapack III Gold) and the phages were replicated in *E. coli* XL1-Blue MRF'. The S cDNA library contained approx. 5×5^5 clones having an average insert size of 1.1 kb, while the B cDNA library contained approx. 6×10^4 clones having an average
30 insert size of 2 kb.

EXAMPLE 3:

RT-PCR using delta-6-desaturase-specific primers

35 By comparing sequences of known desaturases, it was possible to identify conserved regions. While taking into consideration the special ciliate codon usage and/or *Tetrahymena* codon usage, it was possible to

design PCR primers for the particularly strongly conserved amino acid regions

WWKWNHNAHH (SEQ ID No.: 4) and GGLQFQIEHHLFP (SEQ ID No.: 5)

- 5 (Wuitschick JD, Karrer KM (1999) Analysis of genomic G
+ C content, codon usage, initiator codon context and
translation termination sites in *Tetrahymena*
thermophila. J. Eukaryot. Microbiol. 46(3):239-47;
Martindale (1989) J. Protozool. 36,1:29-34, CUTG,
10 (Codon Usage Tabulated from Genbank):

<http://www.dna.affrc.go.jp/~nakamura/CUTG.html>)

Primer 1 (sense): 5'-TGGTGGGAARTGGAMNCAYAA-3', (SEQ ID No.: 6)

- Primer 2 (antisense): 5'-CGDGGRAANARRTGRTGTTC-3' (SEQ
15 ID No.: 7).

- 100 ng of isolated mRNA were employed for the first strand synthesis using AMV reverse transcriptase (Boehringer Mannheim). The reaction took place in accordance with the manufacturer's protocol in a volume of 20 μ l: 50 mM tris-HCl (pH 8.5), 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT, 1 mM dNTPs, 2 pmol of oligo-DT anchor primer (5'-GACCACGCGTATCGATGTCGACT(16)V-3'; SEQ ID No.: 8), 2 units of AMV reverse transcriptase; 60 min at 55°C, and subsequently 10 min at 65°C. 1/10 of this first strand reaction was used for the PCR. The PCR took place in a volume of 25 μ l containing: 1 \times Qiagen HotStarTaq PCR buffer (QIAGEN), pH 8.7 (20°C), 10 pmol each of the delta-6-desaturase-specific primers, in each case 200 μ M dNTPs, 1.5 mM $MgCl_2$, 1 unit of HotStarTaq polymerase (Qiagen). The PCR was carried out under the following conditions: initial denaturation at 95°C for 15 min, with this being followed by 35 cycles consisting in each case of 94°C for 30 sec, 45°C for 30 sec and 72°C for 1 min. In conclusion, 10 min at 72°C. The PCR fragments were ligated into the vector pCR 2.1 by means of T/A cloning (Invitrogen) and replicated in E. coli TOP10F' (Invitrogen). Plasmid DNA was isolated

Genomic DNA was isolated by the urea method (Gaertig et al. 1994) from *Tetrahymena* and cut with Eco R I. The cut DNA was ligated into a lambda vector (Zap Express, 5 Stratagene) which had likewise been cut with Eco R I. Further processing corresponded to the procedure described in the case of the cDNA library.

EXAMPLE 6:

10 Isolating the delta-6-desaturase genomic sequence

The genomic sequence for delta-6-desaturase was investigated by means of PCR. In the first place, a PCR product of approx. 2200 bp in size, which contained the entire coding sequence and introns, was generated from genomic DNA using primers from the 5' and 3' ends of the cDNA:

d6-5'-F: AGTAAGCAAACCTAAATTTAAAAACAAGC (SEQ ID No.:12)

d6-3'-R: GGTCCTTCATGAATCTTAAGGTTCCACTTC (SEQ ID No.:13)

20 The Genome Walker System (Clontech) was used to isolate flanking sequences of the delta-6-desaturase gene. Using the universal primers from this system and specific primers based on the delta-6-desaturase sequence which had been determined, i.e.

25 d6-5'-R: CTTAAGTCTTATCAACTCCCATAATGC (SEQ ID No.: 14)

d6-3'-F: GAAGTGAACCTTAAGATTCATGAAGGACC (SEQ ID No.:15)

it was possible to isolate flanking regions of the Tetrahymena delta-6-desaturase gene. The complete structure of the genomic sequence is depicted in Figure 30 5.

EXAMPLE 7:

Preparing the pBDES6 expression constructs

35 The vector pBICH3 (Gaertig et al. 1999 Nature Biotech.
17:462-465) contains the sequence encoding the
Ichthyophthirius I antigen (G1) preprotein flanked by
noncoding, regulatory sequences of the *Tetrahymena*

WO 01/20000

PCT/EP00/08778

- 30 -

thermophila BTU1 gene. A modified plasmid (pBICH3-Nsi), containing an Nsi I cleavage site at the start (kindly provided by J. Gaertig, University of Georgia, Athens, GA, USA), was used to prepare the delta-6-desaturase expression construct pBDES6. For this, PCR was used to insert Nsi I and Bam HI cleavage sites at the start and stop of the sequences encoding the *Tetrahymena* delta-6-desaturase. Isolated plasmids which contain the complete cDNA sequences for the delta-6-desaturase (pDES6) were employed as the template for the PCR. The primers

D6-Nsi-F: 5'-GCATTATGCATGTTGATAAGACTTAAGAAG-3' (SEQ ID No.: 16)

D6-Bam-R: 5'-TATGGATCCTCAAAGGTGAGATTTTCAAAAATAG-3' (SEQ ID No.: 17)

generated PCR products which contained the complete sequence coding for the delta-6-desaturase flanked by Nsi I and Bam HI cleavage sites. The PCR products, and the plasmid pBICH3-Nsi, were cut with the restriction enzymes Nsi I and Bam HI, purified on an agarose gel and ligated together (see the plasmid construction figure). The resulting pBDES6 expression constructs contained the complete delta-6-desaturase-encoding sequence inserted, in a correct reading frame, into the regulatory sequences of the BTU1 gene (see Figure 6). For transforming *Tetrahymena*, the constructs were linearized by being digested with the restriction enzymes Xba I and Sal I. When successful transformation occurred, the BTU1 gene was replaced with these constructs by homologous recombination, resulting in the cells becoming resistant to paclitaxel.

EXAMPLE 8:

Determining the fatty acid spectrum of the transformants

The fatty acid spectrum was determined using gas chromatographs (HP GC 6890) having a flame ionization

Comparison of the fatty acid spectrum (main fatty acids) of the Tetrahymena pBDES6 transformants (AX601 and AX604) with that of the Tetrahymena wild-type strain (CU522) following 50 h of culture. The table gives the percentage content of the main fatty acids in the total fatty acid spectrum and also the ratio of the unsaturated main fatty acids to the saturated main fatty acids.

WO 01/20000

PCT/EP00/08778

- 33 -

untransformed strain CU522 (Table 1). In addition to the shift in the GLA content, the marked shift in the fatty acid spectrum toward a higher content of unsaturated fatty acids is also striking. In the transformants, the ratio of unsaturated to saturated (main) fatty acids is almost twice as high (Table 2).

EXAMPLE 9:

Preparing the delta-6-desaturase knockout construct
pgDES6::neo

In order to prepare the knockout construct, a neo cassette from the plasmid p4T2-1ΔH3 (Gaertig et al. (1994) Nucl. Acids Res. 22:5391-5398) was inserted into the delta-6-desaturase genomic sequence. This neo cassette is composed of the neomycin resistance gene under the control of the Tetrahymena histone H4 promoter and the 3'-flanking sequence of the BTU2 gene. In Tetrahymena, this construct mediates resistance to paromomycin. The plasmid p4T2-1ΔH3 was cut with Eco RV/ Sma I and the neo cassette fragment, of approx. 1.4 kb in size, was ligated into the Tetrahymena delta-6-desaturase genomic sequence contained in plasmid pgDES6, which had been cut with Eco RV (see Figure 8). This resulted in the plasmid pgDES6::neo. When successful transformation occurred, the gene encoding the delta-6-desaturase was replaced with this construct by means of homologous recombination, resulting in the cells becoming resistant to paromomycin.

EXAMPLE 10:

Macronucleus transformation of Tetrahymena using the desaturase expression construct pBDES6

5×10^6 *Tetrahymena thermophila* cells (CU522) were employed for a transformation. The cells were cultured in 50 ml of SPPA medium at 30°C in a 250 ml Erlenmeyer flask on a shaker at 150 rpm until the cell density had

WO 01/20000

PCT/EP00/08778

- 34 -

reached approx. $3-5 \times 10^5$ cells/ml. The cells were pelleted by centrifuging (1200 g) for 5 min and the cell pellet was resuspended in 50 ml of 10 mM tris-HCl (pH 7.5) and centrifuged as before. This washing step

5 was repeated and the cells were resuspended, at a cell density of 3×10^5 cells/ml, in 10 mM tris-HCl (pH 7.5, plus antibiotics), after which they were transferred to a 250 ml Erlenmeyer flask and incubated at 30°C for 16-20 h without shaking (starvation phase). Following the

10 starvation phase, the cell count was determined once again, after which the cells were centrifuged as above and subsequently adjusted, with 10 mM tris-HCl (pH 7.5), to a concentration of 5×10^6 cells/ml. one ml of the cells was used for the transformation. The

15 transformation was effected by means of microparticle bombardment (see above). For regeneration, the cells were taken up in SSPA medium and incubated at 30°C in an Erlenmeyer flask without shaking. After 3 h, Paclitaxel® was added to give a final concentration of

20 20 μM and the cells were transferred, in aliquots of 100 μl , to 96-well microtiter plates. The cells were incubated at 30°C in a moist, darkened box. After 2-3 days, it was possible to identify Paclitaxel-resistant clones. Positive clones were transferred by inoculation

25 into fresh medium containing 25 μM Paclitaxel. A complete "phenotypic assortment" (Gaertig & Kapler (1999)) was achieved by culturing the cells in increasing concentrations of Paclitaxel (up to 80 μM). In order to analyze the clones, cultures of approx.

30 4 ml were grown in SSPA containing Paclitaxel, after which the DNA was isolated (Jacek Gaertig et al. (1994) FNAS 91:4549-4553) and the DNA which was integrated into the BTU1 locus was amplified by means of PCR. The primers employed were the BTU1-specific primers BTU1-

35 5'F (AAAAATAAAAAAGTTTGAAAAAACCTTC, approx. 50 bp upstream of the start codon, SEQ ID No.: 18) and BTU1-3'R (GTTTAGCTGACCGATTTCAGTTC, 3 bp downstream of the stop codon, SEQ ID No.: 19). The PCR products were

WO 01/20000

PCT/EP00/08778

- 35 -

analyzed on a 1% agarose gel either in the uncut state or having been cut with Hind III or Eco RV (pBDES6) or Eco RI (pBDES9). Complete "phenotypic assortment" was verified by means of RT-PCR using the BTU1-specific
5 primers (Gaertig & Kapler (1999)).

EXAMPLE 11:

Micronucleus and macronucleus transformation of Tetrahymena using the knockout construct pgDES6::neo

10

Tetrahymena strains of differing pairing types (CU428 VII and B2086 II) were cultured separately in SPPA medium, at 30°C and with shaking (150 rpm), in Erlenmeyer flasks. At a cell density of $3-5 \times 10^5$ cells/ml, the cells were centrifuged (1200 g) for 5 min at
15 room temperature. The cells were washed three times with 50 ml of 10 mM tris-HCl (pH 7.5) and finally resuspended in 50 ml of 10 mM tris-HCl (pH 7.5), after which antibiotic solution was added. The cells were
20 incubated at 30°C, without shaking, in Erlenmeyer flasks. After approx. 4 h, the two cultures were counted once again and diluted with 10 mM tris-HCl (pH 7.5) to 3×10^5 cells/ml, after which they were incubated at 30°C for a further 16-20 h. After this
25 starvation phase, the same (absolute) number of cells from the two cultures were mixed in a 2 L Erlenmeyer flask. The cells were incubated at 30°C (beginning of conjugation) and the efficiency of the conjugation was determined after 2 h. For the transformation to be
30 successful, approx. 30% of the cells should be present as pairs at this time.

For the micronucleus transformation, in each case 1×10^7 conjugating cells (5×10^6 pairs) were centrifuged,
35 at 3 h, 3.5 h, 4 h and 4.5 h after the beginning of conjugation, at 1200 g for 5 min and the cell pellet was resuspended in 1 ml of 10 mM tris-HCl (pH 7.5).

For transforming the new macronucleus primordia, cells were centrifuged as above, at 11 h after the beginning of conjugation, and resuspended in tris-HCl. The transformation was effected by means of microparticle bombardment (see above).

For culturing the delta-6-desaturase knockout mutants, 200 µg of borage oil (20-25% GLA; SIGMA)/ml were added to the medium.

10 It was possible to identify transformed cells by selecting for paromomycin resistance. In the case of transforming the micronucleus, paromomycin (final concentration, 100 $\mu\text{g/ml}$) was added at 11 h after beginning the conjugation and the cells were
15 apportioned on 96-well microtiter plates in aliquots of 100 μl . The cells were incubated in a moist box at 30°C. It was possible to identify resistant clones after 2-3 days. It was possible to distinguish genuine micronucleus transformants on the basis of the
20 resistance of macronucleus transformants to 6-methylpurine. When the macronucleus was transformed, paromomycin (final concentration, 100 $\mu\text{g/ml}$) was added approx. 4h after the transformation and the cells were apportioned on 96-well microtiter plates in aliquots of
25 100 μl . The cells were incubated in a moist box at 30°C. It was possible to identify resistant clones after 2-3 days. Positive clones were transferred by inoculation into fresh medium containing 120 μg of paromomycin/ml. A complete "phenotypic assortment"
30 (Gaertig & Kapler (1999)) was achieved after a few generations by culturing the cells in this high paromomycin concentration.

By crossing the micronucleus transformants with a B*VI strain, it was possible to generate homozygous knockout mutants (Bruns & Cassidy-Hanley, Methods in Cell Biology, Volume 62 (1999) 229-240).

EXAMPLE 12:

Biolistic transformation (microparticle bombardment)

Tetrahymena thermophila were transformed by means of
biolistic transformation, as described in Bruns &
5 Cassidy-Hanley (Methods in Cell Biology, Volume 62
(1999) 501-502); Gaertig et al. (1999) Nature Biotech.
17: 462-465) or Cassidy-Hanley et al. ((1997) Genetics
146:135-147). The operation of the Biolistic® PDS-1000/
He Particle Delivery System (BIO-RAD) is detailed in
10 the accompanying manual.

For the transformation, 6 mg of gold particles (0.6 µm;
BIO-RAD) are loaded with 10 µg of linearized plasmid
DNA (Sanford et al. (1991) Biotechniques 3:3-16; Bruns
& Cassidy-Hanley (1999) Methods in Cell Biology, Volume
15 62: 501-512).

Preparation of the gold particles: 60 mg of the 0.6 µm
gold particles (Biorad) were resuspended in 1 ml of
ethanol. For this, the particles were vigorously mixed
3 times, for in each case 1-2 min, on a vortex.
20 Subsequently, the particles were centrifuged (10000 g)
for 1 min and the supernatant was carefully removed
using a pipette. The gold particles were resuspended in
1 ml of sterile water and centrifuged as above. This
washing step was repeated once and the particles were
25 resuspended in 1 ml of 50% glycerol and stored at -20°C
in aliquots of 100 µl.

Preparing the transformation: The macrocarrier holder,
macrocarrier and stopscreens were stored for several
30 hours in 100% ethanol, while the rupture disks were
stored in isopropanol. A macrocarrier was subsequently
inserted into the macrocarrier holder and dried in air.

Loading the gold particles with DNA: All the steps were
35 carried out at 4°C. Gold particles, prepared vector,
2.5 M CaCl₂, 1 M spermidine and 70% and 100% ethanol
were cooled on ice. 10 µl of the linearized vector DNA
(1 µg/ml) were added to 100 µl of prepared gold

particles and the mixture was carefully vortexed for 10 sec. Subsequently, 100 μ l of 2.5 M CaCl_2 were firstly added, after which the mixture was vortexed for 10 sec, and 40 μ l of 1 M spermidine were then added and the
5 mixture was carefully vortexed for 10 min. After adding 200 μ l of 70% ethanol, the particles were vortexed for 1 min and then centrifuged at 10000 g for 1 min. The pellet was resuspended in 20 μ l of 100% ethanol, centrifuged and then resuspended in 35 μ l of 100%
10 ethanol.

The particles which had been prepared in this way were carefully added, using a pipette, to the center of a macrocarrier. The macrocarrier was subsequently stored in a box containing hygroscopic silica gel until the
15 transformation took place.

Transformation: One ml of the prepared cells (see above) was added to the middle of a round filter, which had been moistened with 10 mM tris-HCl (pH 7.5), in a
20 Petri dish and inserted into the lowest slide-in ledge in the Biolistic® PDS-1000/He Particle Delivery System transformation chamber. The transformation took place using the prepared gold particles at a pressure of 900 psi (two 450 psi rupture disks) and under a vacuum
25 of 27 inches of Hg in the transformation chamber. The cells were then immediately transferred to an Erlenmeyer flask containing 50 ml of SPPA medium and incubated at 30°C without shaking.

Patent claims:

1. A nucleic acid encoding *Tetrahymena* delta-6-desaturase having an amino acid sequence as depicted in SEQ ID No.: 2, or a functional variant thereof, and parts thereof containing at least 8 nucleotides, with SEQ ID No.: 1 being part of the claim.
2. A nucleic acid as claimed in claim 1, which is obtained from a ciliate.
3. A nucleic acid as claimed in claim 1 or 2, which is obtained from *Tetrahymena thermophila*.
4. A nucleic acid as claimed in claims 1-3, which is a DNA or RNA, preferably a double-stranded DNA.
5. A nucleic acid as claimed in one of claims 1 to 4, which is a DNA having a nucleic acid sequence as depicted in SEQ ID No.: 1 from position 33 to position 1091.
6. A nucleic acid as claimed in one of claims 1 to 5, which contains one or more noncoding sequences.
7. An isolated nucleic acid as claimed in one of claims 1 to 6 as depicted in SEQ ID No.: 3, or a functional variant thereof, and parts thereof containing at least 8 nucleotides, with SEQ ID No.: 3 being part of the claim.
8. A nucleic acid as claimed in one of claims 1 - 7, which is contained in a vector, preferably in an expression vector.
9. An expression vector as claimed in claim 8, wherein the nucleic acid is functionally combined

with a constitutive and/or inducible promoter and optionally with a termination signal.

- 5 10. A process for preparing a nucleic acid as claimed in one of claims 1-7, which comprises synthesizing the nucleic acid chemically or isolating it from a gene library using a probe.
- 10 11. A polypeptide having an amino acid sequence as depicted in SEQ ID No.: 2 or a functional variant thereof, and parts thereof containing at least 6 amino acids.
- 15 12. A process for preparing a polypeptide as claimed in claim 11, which comprises expressing a nucleic acid as claimed in one of claims 1-7 in a suitable expression system or host organism.
- 20 13. An antibody which is directed against a polypeptide as claimed in claim 11.
14. A transgenic organism which harbors a nucleic acid as claimed in one of claims 1 to 10.
- 25 15. A transgenic organism as claimed in claim 11 which is selected from plants or ciliates.
- 30 16. The use of the nucleic acids as claimed in claims 1-7 and/or polypeptides as claimed in claim 11 for enriching delta-6-desaturase-dependent fatty acids in ciliates.

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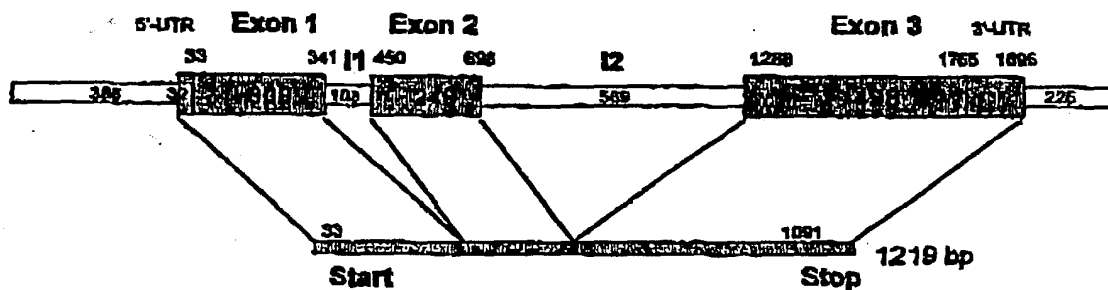
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(54) Title: NUCLEIC ACID WHICH IS OBTAINED FROM TETRAHYMENA AND WHICH CODES FOR A DELTA-6-DE-
SATURASE, THE PRODUCTION THEREOF AND USE(54) Bezeichnung: NUCLEINSÄURE AUS TETRAHYMENA KODIEREND FÜR EINE DELTA-6-DESATURASE, IHRE HER-
STELLUNG UND VERWENDUNG

Struktur des $\Delta 6$ -Desaturase Gens

STRUCTURE OF THE $\Delta 6$ -DESATURASE GENE



(57) Abstract: The invention relates to nucleic acid(s) which is/are obtained from tetrahymena and which code(s) for a ciliate-specific delta-6-desaturase that is involved in the biosynthesis of commercially valuable, multiply unsaturated fatty acids (so-called PUFA: polyunsaturated fatty acids). The inventive nucleotide sequence and the polypeptide sequence that can be obtained therefrom exhibit a surprisingly low sequence identity compared to other known natural desaturases. The invention also relates to the use of the nucleic acid(s) for overexpression in ciliates, preferably tetrahymena, in particular, *Tetrahymena thermophila*, with the aim of increasing the production of delta-6 unsaturated fatty acids, especially GLA.

(57) Zusammenfassung: Die vorliegende Erfindung betrifft Nukleinsäure(n) aus Tetrahymena, die für eine ciliatenspezifische delta-6-Desaturase kodiert, welche an der Biosynthese von kommerziell wertvollen mehrfach ungesättigten Fettsäuren (sog. PUFA engl.: polyunsaturated fatty acids) beteiligt ist. Die erfindungsgemäße Nukleotidsequenz und die daraus erhaltliche Polypeptidsequenz zeigen eine überraschend geringe Sequenzidentität zu anderen bekannten natürlichen Desaturasen. Die Erfindung betrifft ferner die Verwendung der Nukleinsäure(n) zur Überexpression in Ciliaten, vorzugsweise Tetrahymena, insbesondere *Tetrahymena thermophila*, mit dem Ziel einer gesteigerten Produktion von delta-6 ungesättigten Fettsäuren, insbesondere der GLA.

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WO 01/20000 A1

(81) Designated states (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated states (regional): ARIPO Patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ,

TM), European Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

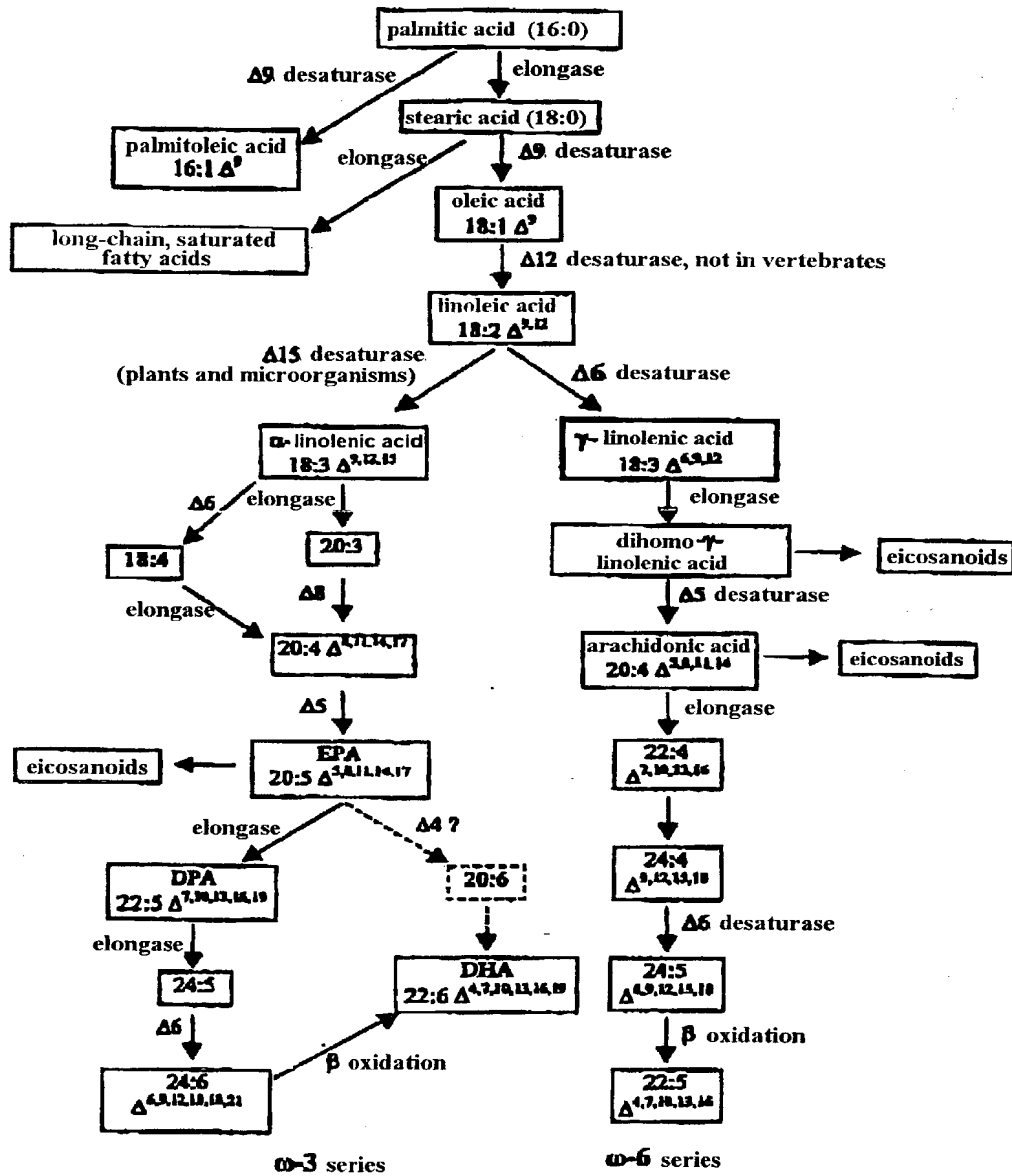
Published:

- *With the International Search Report.*
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For an explanation of the two-letter codes and the other abbreviations, reference is made to the explanations ("Guidance Notes on Codes and Abbreviations") at the beginning of each regular edition of the PCT Gazette.

1/10

Fig. 1



10070610/070666

Fig. 2

BLAST 2.0.0 (Jan-05-1999)
 References: Altschul, Stephen F., Thomas L. Madden, Alejandro A. Schaffer, Jinghui
 5 Zhang, Zheng Zhang, Webb Miller, and David J. Lipman (1997), "Gapped BLAST and PSI-
 BLAST: a new generation of protein database search programs", Nucleic Acids Res.
 25:3389-3402.

Query: T. thermophila, delta-6-Desaturase (352 letters)

Database: /LION/data/db/cant/ordb

367,705 sequences; 119,829,732 total letters

10

15 Sequences producing significant alignments:

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	trembl AF031477 AF031477_1 product: "delta6-fatty-acid-desatura...	79	4e-14
	trembl U81122 CE113F2_1 gene: "T13F2.1"; Caenorhabditis elegans...	78	1e-13
20	trembl Z70271 CEW08D2_2 gene: "W08D2.4"; Caenorhabditis elegans...	78	1e-13
	trembl AF005096 AF005096_1 product: "desaturase/cytochrome b5 p...	70	3e-11
	trembl AJ222980 D7A72980_1 gene: "des6"; product: "delta6-acyl-...	69	6e-11
	trembl U79010 BCU79010_1 product: "delta 6 desaturase"; Borago...	67	2e-10
	trembl AC005397 NC005397_14 gene: "T3F17.16"; product: "putativ...	67	2e-10
25	trembl AF007561 AF007561_1 product: "delta 6-desaturase"; Bora...	66	4e-10
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	trembl new AF126798 AF126798_1 product: "delta-6 fatty acid desa...	63	3e-09

2A/10

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	trembl new AB021980 AB021980_1 product: "delta-6 fatty acid desat...	62 6e-09
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	trembl AC004770 AC004770_2 product: "BC269730_2", Homo sapiens...	60 3e-08
	trembl AJ224161 ATM04161_1 gene: "old1", product: "delta-8 sphi...	59 6e-08
	trembl new AL050110 HSM00210_1 gene: "MMP036C201", product: "...	57 2e-07
35	trembl AB022097 AB022097_1 product: "delta 5 fatty acid desatur...	57 2e-07
	trembl X87143 HACTYBSNV_1 product: "cytochrome b5 containing fa...	50 2e-05
	trembl X08460 HMF028_1 gene: "Hden", product: "Hden protein", ...	50 3e-05
	trembl AF001394 AF001394_1 product: "fatty acid desaturase/cyto...	46 4e-04
	trembl AF002669 H9A12669_1 product: "HLD", Homo sapiens putati...	46 5e-04
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Continuation of Fig. 2

3/10

Fig 3A

[illegible]


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DE
FILE

[illegible]

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四**

[illegible]

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[illegible][illegible]

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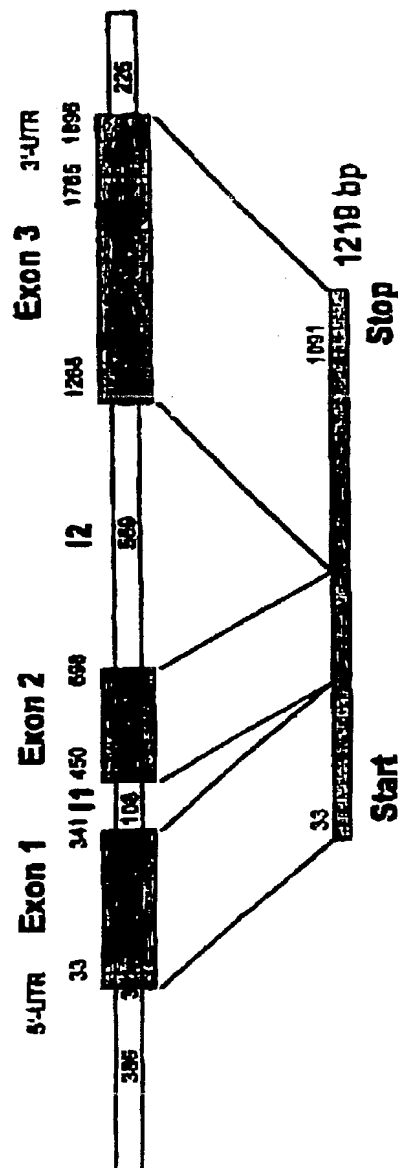
Fig. 4

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	B. officinalis	1	-----NDAQIKYITUS	-----P	-----P	-----D
	M. musculus	1	-----NDAQIKYITUS	-----P	-----P	-----D
	T. thermophila	1	-----NDAQIKYITUS	-----P	-----P	-----D
10	M. alpina	54	-----SVT	-----P	-----P	-----D
	C. elegans	54	-----SVT	-----P	-----P	-----D
	B. officinalis	45	-----P	-----P	-----P	-----D
	M. musculus	57	-----P	-----P	-----P	-----D
	T. thermophila	46	-----P	-----P	-----P	-----D
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	B. officinalis	91	-----EAA	-----P	-----P	-----D
	M. musculus	105	-----KSSQIT	-----P	-----P	-----D
	T. thermophila	92	-----K	-----P	-----P	-----D
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	C. elegans	146	-----P	-----P	-----P	-----D
	B. officinalis	133	-----P	-----P	-----P	-----D
	M. musculus	155	-----P	-----P	-----P	-----D
	T. thermophila	126	-----P	-----P	-----P	-----D
25	M. alpina	199	-----P	-----P	-----P	-----D
	C. elegans	205	-----P	-----P	-----P	-----D
	B. officinalis	186	-----P	-----P	-----P	-----D
	M. musculus	207	-----P	-----P	-----P	-----D
	T. thermophila	174	-----P	-----P	-----P	-----D

Continuation of Fig. 4

Fig. 5

Structure of the Δ -6-desaturase gene



Preparation of the pBDES6 expression construct

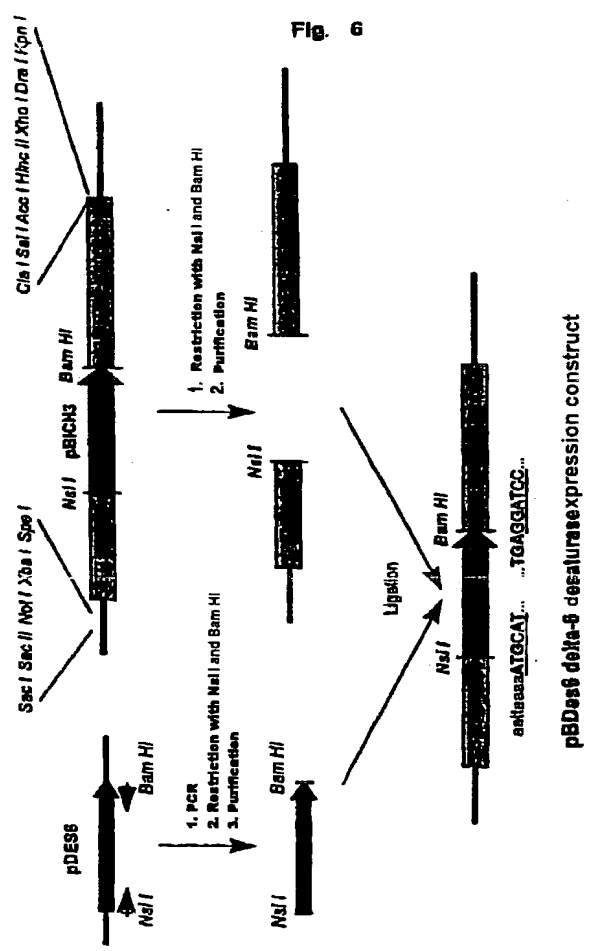
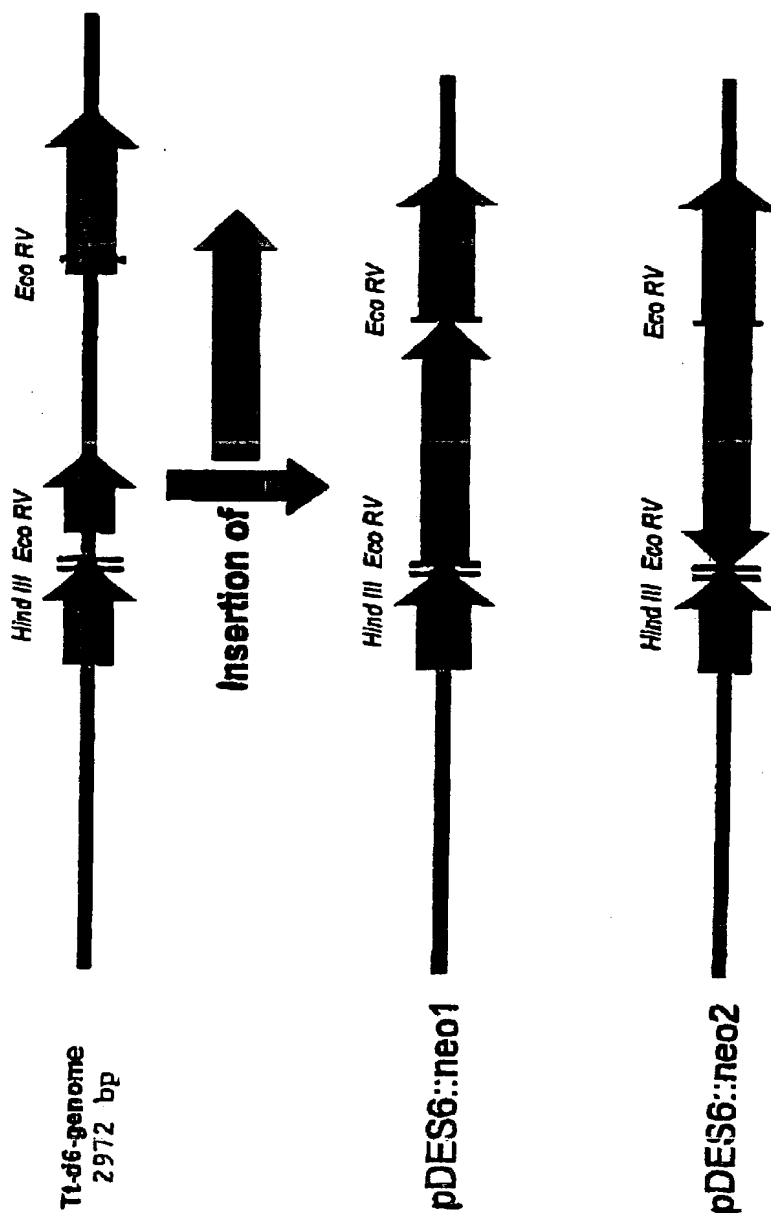


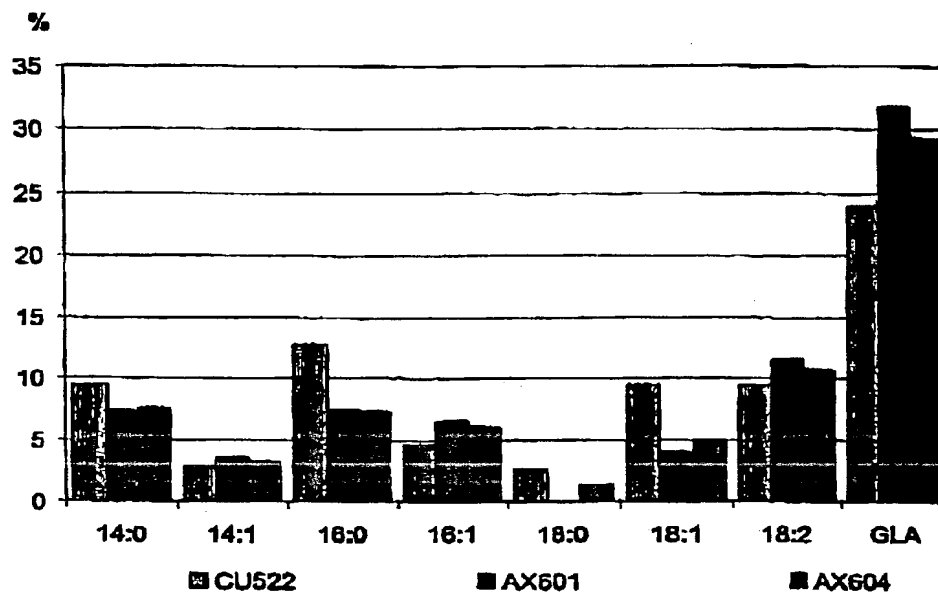
Fig. 7

Delta-6-desaturase knockout construct



10/10

Fig. 8



Comparison of the fatty acid spectrum (main fatty acids) of the Tetrahymena pBDES6 transformants (AX601 and AX604) with that of the Tetrahymena wild-type strain (CU522) after 50 h of culture. The figure gives the percentage content of the main fatty acids in the total fatty acid spectrum

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PTO/SB/01 (12-97)

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DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)	Attorney Docket Number	005430.00002
	First Named Inventor	Rusing, Matthias
	COMPLETE IF KNOWN	
	Application Number	10/070,666
	Filing Date	March 8, 2002
	Group Art Unit	
<input type="checkbox"/> Declaration Submitted with Initial Filing	OR	<input checked="" type="checkbox"/> Declaration Submitted after Initial Filing (surcharge (37 CFR 1.16 (e)) required)
Examiner Name		

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Nucleic Acid Which Is Obtained From Tetrahymena And Which Codes For A Delta-6-Desaturase, The Production Thereof And Use

the specification of which

☐ is attached hereto

OR

☒ was filed on (MM/DD/YYYY) **March 8, 2002** as United States Application Number or PCT International Application Number **10/070,666** and was amended on (MM/DD/YYYY) (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119(a)(1) or 356(b) of any foreign application(s) for patent or inventor's certificate, or 356(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Priority Not Claimed	Certified Copy Attached?	
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PCT/EP00/08778 199 43 270.8	PCT DE	09/08/2000 09/10/1999	<input type="checkbox"/>	<input type="checkbox"/>	<input checked="" type="checkbox"/>

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) filed before:

Application Number(s)	Filing Date (MM/DD/YYYY)

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

[Page 1 of 2]

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DECLARATION — Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 35 U.S.C. 122 of any PCT international application designating the United States of America, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior information which is material to patentability as defined in 37 CFR 1.55 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Parent Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/EP00/08778	September 8, 2000	

☐ Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02C attached hereto.

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Address			
City	State	ZIP	
Country	Telephone	Fax	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: ☐ A petition has been filed for this unsigned inventor

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Inventor's Signature [Signature] Date 7.08.02

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Post Office Address 50935

City Koln State ZIP Country Germany

☒ Additional inventors are being named on the 1 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto

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DECLARATION

ADDITIONAL INVENTOR(S)
Supplemental Sheet
Page 1 of 1

Name of Additional Joint Inventor, if any:

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Kiv

Inventor's
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1/8

PCT/EP00/08778

SEQUENCE LISTING

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WO 01/20000

PCT/EP00/08778

2/8

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WO 01/20000

PCT/EP00/08778

4/8

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 <213> Tetrahymena thermophila

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<210> 5
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 <213> Tetrahymena thermophila

<400> 5
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 1 5 10

WO 01/20000

PCT/EP00/08778

5/8

<210> 6
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<212> DNA
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<220>

<223> Description of the artificial sequence:primer

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20

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<223>Description of the artificial sequence:primer

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20

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<223> Description of the artificial sequence:primer

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<223> Description of the artificial sequence:primer

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28

WO 01/20000

PCT/EP00/08778

7/8

<210> 14
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<220>

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<212> DNA
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<223> Description of the artificial sequence:primer

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35

WO 01/20000

8 / 8

PCT/EP00/08778

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